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Inventors: William H. Velander  
Susan T. Lord  
William N. Drohan  
Henryk Lubon  
John L. Johnson  
Christopher G. Russell

## TRANSGENIC FIBRINOGEN

### BACKGROUND OF THE INVENTION

Inc B<sup>1</sup>

The present invention relates in general to the use of transgenic animals to produce therapeutically useful amounts of clinically important recombinant proteins.

5 More particularly, this invention relates to the production in transgenic animals of clinically useful quantities of the blood clotting protein, fibrinogen ("FIB").

10 The ultimate event in the blood clotting cascade is the thrombin-catalyzed conversion of FIB (Mr=340,000) to fibrin (Mr=329,000), the latter forming the fibrin clot. FIB deficiency is generally transmitted as an autosomal recessive trait and may manifest as a complete or partial absence of FIB from the blood plasma. Clinically, the  
15 disease resembles moderate or mild hemophilia. Congenital fibrinogen abnormality may be due to the hereditary synthesis of structurally or functionally abnormal molecules, as in Vlissingen, Ijmuiden and Nijmegen fibrinogens. An acquired deficiency of this  
20 protein may occur due to impaired hepatic synthesis of the protein, as occurs, for example, in hepatitis or hepatic necrosis, or to accelerated destruction of the protein caused, for example, by increased blood proteolytic activity.

25 Control of bleeding in such patients is currently achieved by transfusion of FIB contained in freshly-frozen human plasma or in concentrates of the protein isolated from donor blood. While these replacement therapies are generally effective, they place patients at  
30 risk for virus-transmissible diseases such as hepatitis

or AIDS. Although this risk has been greatly reduced by inactivating such viruses with heat or organic solvents, such preparations have greatly increased the cost of treatment, and are not risk free. There is thus a  
5 critical need for a source of this protein alternate to human plasma.

An important advance in obtaining an alternate clinical source of FIB has been the cloning of cDNAs encoding the three different fibrinogen chains, and the  
10 publication of cDNA sequences. Rixon et al., *Biochemistry* 22: 3237 (1983); Chung et al., *ibid*: 3244; Chung et al., *ibid*: 3250. The structure of the FIB molecule is exceedingly complex. Each molecule of FIB consists of two sets of three different polypeptide  
15 chains, designated A $\alpha$ , B $\beta$  and G $\gamma$ , with molecular masses of 66 kDa, 52 kDa and 46.5 kDa, respectively. The two half-molecules containing each set of chains are linked together by three disulfide bonds. In addition, a complex set of intra- and inter-chain disulfide bonds  
20 (there are a total of 29 disulfide bonds with no free sulfhydryl groups) are involved in maintaining proper functional structure. Further, FIB is a glycoprotein with highly specific glycosylations. The molecule contains four carbohydrate chains, one each on the B,  $\beta$ ,  
25 G and  $\gamma$  chains; the  $\alpha$  and A chains contain no carbohydrate. About 11 kDa of the total molecular mass of FIB (340 kDa) is attributable to this carbohydrate, added to the molecule post-translationally. In addition, isoforms of glycoproteins are known corresponding to  
30 differences in sialic acids on the carbohydrate chains. Proper carbohydrate modification is required for functional activity of FIB.

These highly complex characteristics of the functional FIB molecule has made unpredictable and  
35 difficult the expression, assembly and secretion of fully formed and functional recombinant molecules. A cDNA encoding the human FIB A $\alpha$  chain has been expressed in

bacteria. Lord, *DNA* 4:33 (1985). This is of limited usefulness, however, since the other fibrinogen chains that bear carbohydrates cannot be produced in prokaryotes.

5 Individual FIB chains have been expressed in COS1 (transformed monkey kidney fibroblast) cells. Danishevsky et al., *Biochim. Biophys. Acta* 1048: 202 (1990). In addition, transfecting COS1 cells with a combination of cDNAs encoding the individual human  
10 fibrinogen subunit chains is reported to produce the holoprotein, but the amounts produced were small, and substantially less than the production achieved in the transgenic animal systems to be described below. Roy et al., *J. Biol. Chem.*, 266: 4758 (1991). The secretion of  
15 partially assembled or wholly unassembled and separate human FIB ("hFIB") or recombinant human FIB ("rhFIB") chains has not been reported for native or genetically engineered tissues. Chung et al. (1983); Danishevsky et al (1990). In addition, there are serious drawbacks to  
20 the use of mammalian cell tissue culture systems for production of FIB. These include the high costs of growth media, the labor intensive nature of such systems, and limited production capacity.

An important need persists for an efficient and  
25 relatively inexpensive means of producing clinically useful amounts of infectious particle-free rhFIB protein. The present invention satisfies this need. It has been surprisingly found that transgenic animals can be genetically engineered to produce and secrete into  
30 readily accessible body fluids therapeutically useful quantities of rhFIB. In addition to therapeutic uses involving replacement or addition therapy, the FIB of the invention finds us in a variety of applications, such as a "glue" in surgical procedures, as a delivery system for  
35 drugs, such as antibiotics or anti-parasitic agents, to wounds, as a food substitute, and for altering the

composition of milk. These transgenic systems are described below.

#### SUMMARY OF THE INVENTION

5 In accordance with the present invention, there is provided a non-human mammalian animal that has stably integrated into its genome heterologous (that is, exogenously derived) polynucleotides that encode the A $\alpha$ , BB and G $\gamma$  polypeptide chains of human FIB, and that direct by means of regulatory and signal sequences the expression of biologically active rhFIB in mammary gland cells, such that newly synthesized fibrinogen is secreted into bodily fluid compartments, particularly milk, blood or urine of the animal. By integrating fewer than all of the three heterologous polynucleotides, individual chains of fibrinogen can be produced and may be secreted. By 10 integrating heterologous polynucleotides that have been modified prior to administration to the host animal, modified FIB and products thereof can be produced. 15

It therefore is an object of the present invention 20 to provide transgenic animals capable of producing rhFIB, individual polypeptide subunit chains of FIB, or FIB-derived proteins and protein products.

It is also an object of the invention to provide a means of producing rhFIB, individual polypeptide subunits thereof, or FIB-derived proteins and protein products in 25 transgenic animals.

In a preferred embodiment of this aspect of the invention, lactating transgenic animals produce the rhFIB, individual FIB subunits or FIB-derived proteins in 30 their mammary glands and secrete these products in their milk. In another preferred embodiment, transgenic animals secrete the produced rhFIB, individual FIB subunits, or FIB-derived proteins in their blood and/or urine.

**BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 is a sketch of the murine WAP gene.

Figure 2 is a sketch of the plasmid pUCWAP4 cassette vector containing ~ 2.6 kbp of WAP 5' promotor region,  
5 ~1.3 kbp of WAP 3' UTR and flanking 3' region.

Figure 3 is a sketch of the plasmid pUCWAP5 cassette vector containing the WAP fragment of pUCWAP4 with added *Not I* linkers.

Figure 4 is a sketch of the pUCWAP5 plasmid  
10 incorporating a polynucleotide encoding the FIB A $\alpha$  1 chain.

Figure 5 is a sketch of the pUCWAP5 plasmid incorporating a polynucleotide encoding the FIB B $\beta$  1 chain.

Figure 6 is a sketch of the pUCWAP5 plasmid  
15 incorporating a polynucleotide encoding the FIB G $\gamma$  1 chain.

Figure 7 shows the family tree of transgenic mice that have incorporated into their genome DNAs encoding  
20 FIB genes.

Figure 8 is a schematic of the means for separating endogenous from rhFIB in mouse milk whey and plasma.

Figure 9 shows a Western blot under non-reducing conditions of transgenic rhFIB in mouse whey and plasma.

Figure 10 shows the same experiment as in Figure 9,  
25 except that the gel was run under reducing conditions.

Figure 11 shows an SDS-PAGE separation of rhFIB after digestion with thrombin.

Figure 12 shows the results of assays for  
30 thrombinopeptide A after digestion by thrombin of rhFIB.

**DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

An approach has been discovered to producing non-human, transgenic mammalian animals that are genetically engineered to secrete, into readily accessible body

fluids such as milk, blood and urine, recombinant holofibrinogen (for example, rhFIB), individual subunit chain polypeptides of FIB, and modified FIB subunit chains in amounts and in forms that are suitable for treating humans with genetic or acquired deficiencies of the normal protein.

#### Glossary

The term "animal" here denotes all mammalian animals except humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages. A "transgenic" animal is any animal containing cells that bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by microinjection or infection with recombinant virus.

"Transgenic" in the present context does not encompass classical crossbreeding or *in vitro* fertilization, but rather denotes animals in which one or more cells receive a recombinant DNA molecule. Although it is highly preferred that this molecule be integrated within the animal's chromosomes, the present invention also contemplates the use of extrachromosomally replicating DNA sequences, such as might be engineered into yeast artificial chromosomes.

The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic information has been taken up and incorporated into a germ line cell, therefore conferring the ability to transfer the information to offspring. If such offspring in fact possess some or all of that information, then they, too, are transgenic animals.

The information to be introduced into the animal is preferably foreign ("heterologous") to the species of animal to which the recipient belongs, but the information also may be foreign only to the particular individual recipient, or genetic information already

possessed by the recipient. In the last case, the introduced gene may be differently expressed than is the native gene.

5 By "holofibrinogen" is meant the mature, fully glycosylated, physiologically functional fibrinogen. Unless specified otherwise, the abbreviations FIB or hFIB or hrFIB as used herein will refer to this holoprotein.

Animals

10 The transgenic animals of this invention may be any animal, other than human, that produces milk, blood serum, and urine. Farm animals (pigs, goats, sheep, cows, horses, rabbits and the like), rodents (such as mice), and domestic pets (for example, cats and dogs) are included in the scope of the present invention.

15 Stable Integration of DNA Constructs in Animal Genome

It is highly preferred that the transgenic animals of the present invention be produced by introducing into single cell embryos appropriate polynucleotides that encode human holoFIB, or individual subunit chain polypeptides or modified products thereof, in a manner such that these polynucleotides are stably integrated into the DNA of germ line cells of the mature animal and inherited in normal mendelian fashion.

25 Advances in technologies for embryo micromanipulation now permit introduction of heterologous DNA into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal. In a preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo. In a most preferred method, however, the appropriate DNAs are coinjected into the pronucleus or cytoplasm of embryos,

preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals. Those techniques as well known. For instance, reviews of standard laboratory procedures for microinjection of heterologous DNAs into mammalian (mouse, pig, rabbit, sheep, goat, cow) fertilized ova include: Hogan et al., MANIPULATING THE MOUSE EMBRYO, Cold Spring Harbor Press, 1986; Krimpenfort et al., *Bio/Technology* <sup>9: 844</sup> ~~9: 86~~ (1991); Palmiter et al., *Cell* 41: 343 (1985); Kraemer et al., GENETIC MANIPULATION OF THE EARLY MAMMALIAN EMBRYO, Cold Spring Harbor Laboratory Press, 1985; Hammer et al., *Nature*, 315: 680 (1985); Purcel et al., *Science*, 244: 1281 (1986); Wagner et al., U.S. patent No. 5,175,385; Krimpenfort et al., U.S. patent No. 5,175,384, the respective contents of which are incorporated by reference. The human FIB or FIB subunit genes can be obtained by isolating them from an appropriate genomic source (i.e., human liver, which is the natural organ for production of this protein) by alternate methods which include preparation of cDNAs from isolated mRNA templates, direct synthesis, or some combination thereof. The cDNAs encoding individual FIB chains can be fused, in proper reading frame, with appropriate regulatory signals as described in detail below, to produce a genetic construct that is then amplified, for example, by preparation in a bacterial vector, according to conventional methods. See, for example, the standard work: Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Press, 1989, the contents of which are incorporated by reference. The amplified construct is thereafter excised from the vector and purified for use in producing transgenic animals. Purification can be accomplished by means of one or more cycles of anionic HPLC; alternate techniques include ultracentrifugation through a sucrose or NaCl gradient, gel electroelution followed by agarose treatment and ethanol precipitation, or low pressure chromatography.



Purification by several cycles of HPLC allows for remarkably high transformation frequencies, on the order of 20% or more in both mice and pigs.

5 Although the present invention preferably entails the use of DNA constructs that produce the desired or native human FIB *per se*, the desired protein may also be produced as a fusion protein containing another protein. For example, the desired recombinant FIB protein of this invention may be produced as part of a larger recombinant  
10 protein in order to stabilize the desired protein or to make its purification from milk faster and easier. The fusion partners are then separated chemically or enzymatically, and the desired FIB isolated. Production of a fusion product between FIB and  $\beta$ -lactoglobulin (BLG) or between FIB and another milk protein are contemplated.  
15

The rhFIB may also be produced having the identical sequence as the native molecule, or it may be produced as a fragment or derivative. A variety of modified rhFIB or subunits thereof can be produced by altering a cloned DNA using the well-known techniques of *in vitro* mutagenesis  
20 such as those set out in the references above.

Production of transgenic animals containing the gene for human FIB involves the use of cDNA or genomic DNA that encodes the  $\alpha$ ,  $\beta$ , and  $\gamma$  chains of hFIB. See Rixon  
25 *et al.*, 1983, and Chung *et al.*, 1983, *supra*. The full-length base sequence of each chain is provided in these references, the contents of which therefore are incorporated by reference.

DNA constructs useful in the present invention  
30 provide a double stranded DNA sequence encoding FIB operably linked to all of the *cis*-acting signals necessary for mammary gland-specific expression of this protein, post-translational glycosylation of two of the three sets of chains of FIB ( $\beta\beta$  and  $G\gamma$ ), secretion of FIB  
35 into milk or other body fluids, and expression of full biological activity. As set out above, polynucleotides encoding FIB or FIB subunit chains suitable for use in

the invention can be isolated by standard techniques starting from the known polynucleotide sequences.

Modified FIB DNA sequences also can be employed in this invention. Useful modifications within this context include, but are not limited to, those that alter post-translational modifications, size or active site of FIB, or that fuse this protein or portions thereof to another protein. Such modifications can be introduced into the protein by techniques well known in this art, such as by synthesizing modified genes by ligation of overlapping oligonucleotide or introducing mutations into the cloned genes by, for example, oligonucleotide-mediated mutagenesis. See, for example, Ebert et al., *Bio/Technology* 9: 835 (1991), Denman et al., *loc. cit.*: 839 (1991), and Gerard et al., *Science* 248: 1421 (1991), for details of procedures for site mutagenesis of proteins. One or more FIP glycosylation sites can be eliminated or changed by site mutagenesis, for instance. Another variant might be a chimeric FIB having a tissue growth factor as protease inhibitor peptide attached to the nascent FIP polypeptide during transgenic biosynthesis or by co-current translation. The assembly of the complex hexameric structure may be exploited to include a guest molecule, but while maintaining recognition of the FIB complex for secretion. Specific changes of entire domains within FIB could be used to impact specialized activity of other molecules, but still remain the hexameric structure required for secretion.

The *cis*-acting regulatory regions useful in the invention include the promoter that drives expression of the FIB or FIB subunit chain genes. Highly preferred are promoters that are specifically active in mammary gland cells and that involve milk proteins. Among such promoters, highly preferred are the whey acidic protein (WAP), short and long  $\alpha$ ,  $\beta$  and kappa casein,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin ("BLG") promoters.

Promoters may be selected on the basis of the protein compositions of various milks.

For example, the WAP and BLG promoters are particularly useful with transgenic rodents, pigs and sheep. The rodent WAP short and long promoters have been used to express the rat WAP gene, the human tPA gene and the CD4 gene, while the sheep BLG promoter has been used to express the sheep BLG gene, the human alpha-1-antitrypsin gene and the human Factor IX gene. For reviews, see Paleyanda et al. in RECOMBINANT TECHNOLOGY IN HEMOSTASIS AND THROMBOSIS (eds. Hoyer et al.), Plenum Press, NY (1991), at page 197; Clark et al., *TIBTECH* 5: 20 (1987), the respective contents of which are incorporated by reference. Preferred among the promoters for carrying out the present invention are the rodent casein and WAP promoters (see, for example, Rosen, U.S. patent No. 5,304,489, and Meade, U.S. patent No. 4,873,316) and the casein,  $\alpha$ -lactalbumin and BLG promoters (Strijker et al., U.S. patent nos. 5,322,775 and 5,366,864) from porcine, bovine, equine and ovine (pigs, sheep, goats, cows, horses), rabbits, rodents and domestic pets (dogs and cats); accordingly, the respective contents of the aforementioned patents are incorporated by reference. The genes for these promoters have been isolated and their characterizations published. For reviews see Clark et al. (1987), *supra*, and Henninghausen, *Protein Expression and Purification* 1: 3 (1990). The mouse WAP promoter is commercially available; see ATCC catalogue no. 63005 (2d ed., 1991), Rockville, MD.

The promoter may be isolated by carrying out conventional restriction endonuclease and subcloning steps. A preferred mouse WAP promoter can be isolated as a 2.6 kb *EcoRI*-*KpnI* fragment which is immediately 5' to the WAP signal sequence. The "long" WAP promoter -- the 5' 4.2 kb *Sau3A*-*KpnI* promoter of the mouse WAP gene, or a fragment thereof -- is also suitable for carrying out

the present invention (see, Paleyanda et al, 1991 above; Henninghausen, 1990, above).

5 Important to the present invention are regulatory sequences that direct secretion of proteins into milk and/or other body fluids of the transgenic animal. In this regard, both homologous and heterologous regulatory sequences are useful in the invention. Generally, regulatory sequences known to direct the secretion of milk proteins, such as either signal peptides from milk  
10 for production of individual FIB subunit chain polypeptides or the nascent target polypeptide for production of the holoFIB, can be used. Signal sequences also can be used in accordance with this invention that direct the secretion of expressed proteins into other  
15 body fluids, particularly blood and urine, such as signal sequences for coagulation proteins such as protein C and Factor VIII.

Unexpectedly, it has been found that a trigenic animal can efficiently express three separate transgenes  
20 which do not have a signal peptide operably linked between promoter and encoding DNA sequences, but still direct synthesis and secretion of a assembled recombinant human fibrinogen protein in milk of a transgenic animal. This occurs in a temporally regulated way as to enable a  
25 complex asymmetric assembly of the fibrinogen polypeptides which nascent polypeptides then enables subsequent secretion of the hexameric, 340 kDa protein in milk. It is important to note that, in general, mammalian cells do not efficiently secrete separate  
30 fibrinogen chains. See Danishefsky et al., *Biochim. Biophys. Acta* 1048: 202 (1990), and Hartwig et al., *J. Biol. Chem.*, 266: 6578 (1991). This phenomenon has been explained by Danishefsky et al. to be a consequence of proper folding rather than the presence of a specific  
35 domain within one of the component chains that may be a prerequisite for fibrinogen secretion; this, since neither of the three chains of fibrinogen by themselves

have the integral signal for secretion. A separate, identifiable signal sequence therefore does not appear to exist in any of the individual  $\alpha$ ,  $\beta$  and  $\gamma$  polypeptides (and, hence, there is no signal sequence encoded immediately preceding the coding sequence for any of the nascent polypeptides in the cDNA or genomic sequences of fibrinogen chains) for directing secretion. Importantly, it has been discovered that it is not necessary to add any coding <sup>sequence</sup> for milk protein or other specific signal sequences to the fibrinogen cDNAs used to make transgenic mice, pigs and cows which secrete properly folded and active transgenic hFIB. The highly preferred constructs consist of three separate transgenes; each transgene has the 2.5 kb-WAP-promoter ligated directly to the native cDNA for one the  $\alpha$ ,  $\beta$ , and  $\gamma$  FIB polypeptides with no intervening signal sequence.

As noted above, there is no requirement for a coding DNA for signal peptide sequences in each of the present three FIB polynucleotide constructs when producing holoFIB. The prior art does not suggest this fact, nor does it <sup>present</sup> ~~presage~~ a properly folded, complex recombinant fibrinogen structure which specifically enables the mammary tissue Golgi transport system to efficiently secrete the assembled FIB. The present examples have three salient features which show that indeed the mammary tissue can perform such a task. First, the levels of rFIB found are consistent with the stable expression levels found for prior examples of 2.5 kb 5'-WAP promoter-cDNA single gene, single polypeptide expressions in the milk of transgenic mice (i.e., about 1-100  $\mu$ g/ml; Hennighausen et al., above). Second, the transgenic mice secrete fibrinogen without involution as indicated by their normal lactation and pup weight gain. Third, the recombinant fibrinogen secreted is physiologically active. Thus, in combination, these results suggest that no significant intracellular buildup of assembled fibrinogen occurs in the mammary epithelium which is

sufficient to halt lactation. Likewise, no appreciable secretion of individual unassembled polypeptides occurred in trigenic- $\alpha, \beta, \gamma$ -fibrinogen mice.

5 In contrast to the trigenic animal, it appears that a signal peptide is needed for each cDNA sequence encoding  $\alpha$ ,  $\beta$  and  $\gamma$  if the unassembled, individual chains are to be efficiently secreted from the mammary epithelium.

10 Among the useful sequences that regulate transcription, in addition to the promoters discussed above, are enhancers, splice signals, transcription termination signals, and polyadenylation sites. Particularly useful in this regard are those that increase the efficiency of the transcription of the genes  
15 for fibrinogen in the mammary gland cells of the transgenic animals listed above. Preferred are transcription regulatory sequences for proteins highly expressed in the mammary gland cells (see above).

20 Preferably, the expression system or construct of this invention also includes a 3' untranslated region downstream of the DNA sequence encoding the desired recombinant protein, or the milk protein gene used for regulation. This region apparently stabilizes the RNA transcript of the expression system and thus increases  
25 the yield of the desired protein. Among the 3' untranslated regions useful in this regard are sequences that provide a poly A signal. Such sequences may be derived, for example, from the SV 40 small t antigen, from the casein 3' untranslated region, and from other 3'  
30 untranslated sequences well known in this art. Preferably, the 3' untranslated region is derived from a milk-specific protein. The stabilizing effect of this region's poly A transcript is important in stabilizing the mRNA of the expression sequence. Also important in  
35 increasing the efficiency of expression of FIB are ribosome binding sites. Likewise, sequences that

regulate the post-translational modification of FIB subunits are useful in the invention.

5 Pursuant to the present invention, therefore, a double-stranded chimeric DNA, including genes that code for the FIB subunit chains and that are operably linked to *cis*-acting regulatory sequences which promote efficient expression of the former in milk and/or other body fluids, are introduced into an embryo, where they are integrated into the embryonic genome and become part of the inheritable genetic endowment of all of the animals cells, including the germ line cells, of the adult which matures from the embryo. The FIB thus expressed and secreted into milk is immunologically reactive, as measured by an ELISA assay described below.

10 Where the synthesis of a FIB subunit chain may be limiting in the production of the holoprotein, expression of this chain can be increased by placing the gene in a different genomic locus. This other locus can contain a DNA sequence under the same or a different regulatory sequence than the other two FIB sequences.

15 In a particularly preferred embodiment the transgenes of the invention generally consist of milk protein upstream and downstream and flanking sequences in combination, separately, with each of the three translated portions of the cDNA sequences representing the three FIB subunit chains. A native 5'-WAP regulatory sequence ending in an accessible restriction site immediately before/at the ATG codon may be ligated to each of the respective restriction sites which occur at the ATG of translatable sequences with no linker sequences derived from the chains of human FIB. A restriction site may be made through site mutagenesis of three bases located in each 3'-untranslatable sequences of the three FIB cDNAs. This preserves the stop sequence while adding a restriction site immediately after each of the translatable sequences of the FIB cDNAs. Each of the combined 5'-regulatory and FIB translatable sequences

which end in a particular restriction site then can be ligated to a corresponding restriction site which occurs at the beginning of the 3'-untranslated region of WAP and adjoining WAP 3'-flanking region. This construction  
5 motif enables native 5'-regulatory and 3'-UTR of the milk protein genes to be immediately juxtaposed without intervening sequences (but see above relating to the insertion of the B $\beta$  gene at a different locus). Particular restriction sites at the ends of all  
10 constructs may be selected in order to facilitate concatenation of A $\alpha$ , B $\beta$  and G $\gamma$  constructs into a single domain within the animal genome.

Although the foregoing, general description of the DNA constructs within the present invention relates in  
15 several places to the WAP promoter, it is emphasized that other suitable promoters (see above for discussion) can be ligated to the fibrinogen encoding polynucleotides in a similar manner. By way of illustration, the following discussion describes the use of the bovine lactoglobulin  
20 ("BLG") promoter to increase the efficiency of expression of FIB and FIB-BLG fusion proteins in mammary glands.

Using techniques described above, fibrinogen encoding cDNA may be inserted into an ovine BLG gene. In order to produce such a construction, for instance, the 11.2 Kbp  
25 ovine BLG gene may be modified to possess a unique EcoRV site upstream of the initiator ATG codon in the vector pUCXSRV. The sequence around this region is changed as follows:

30  
7070  
Seq. ID No. 1 Natural PvuI MetLys  
CCCCAGCTGCAGCCATGAAG  
Seq. ID No. 2 pUCXSRV EcoRV MetLys  
CCCCAGGGATATCCCTGCAGCCATGAAG

This allows the cloning of blunt end fragments upstream of the BLG gene. The 1.5 kbp fragment from a  
35 plasmid (e.g., pWAP FIB) which contains a cDNA encoding human fibrinogen is isolated, blunt ends are generated with T4 DNA polymer, and the product is ligated to



EcoRV-cleaved pUCXSRV. Following transformation of *E. coli* with this plasmid, clones that are produced can be characterized by restriction analysis of plasmid DNA prepared by a mini-prep method and by determination of the nucleotide sequence around the 5' and 3' cloning junctions for the DNA. Clones having the desired structure can be used to produce transgenic rodents, pigs, sheep, cows, horses and other farm animals and domestic pets (cats and dogs) that secrete a FIB-BLG fusion product into their biological fluids as described below.

A hFIB genomic sequence also may be fused to the ovine BLG promoter illustrated in the following discussion. DNA sequences encoding ovine BLG in plasmid pUCXSRV are deleted to generate a vector containing only ovine BLG promoter sequences(pUCSV). As with pUCSRV, blunt-ended fragments can be fused to this promoter region by ligation to a unique EcoRV site. The sequences 5' to this site are identical in both plasmids.

Genomic FIB sequences of greater than about 15 kbp can be introduced into transgenic animals, despite their length, through the use of cosmids with overlapping FIB sequences, whereupon the necessary assembly of an entire genomic polynucleotide encoding hFIB is achieved by homologous recombination *in vivo* after microinjection into an embryo cell. In constructs useful in the foregoing example, a plasmid in which the FIB genomic sequences are fused to ovine BLG 3' flanking sequences just after the fibrinogen translation termination codon to ensure proper transcription, termination and polyadenylation. The hFIB gene fused to ovine BLG 3' flanking sequences is excised from the plasmid, the 3' overhangs repaired using Klenow enzyme, and the product ligated to EcoRV-cleaved pUCSR. Following transformation of *E. coli*, the resulting clones are characterized by restriction DNA analysis and by determining the nucleotide sequences around the 5' and 3' cloning

junctions. Clones having the desired structure may be introduced into fertilized animal ova for production of transgenic animals.

5 A variety of vectors based on the BLG gene may be constructed. In constructs based on this approach, the sequences encoding the ovine BLG protein are deleted, but the 5' promoter sequences are retained. Each may possess a different complement of introns from the ovine gene and a unique EcoRV site allowing the cloning of blunt ended  
10 fragments between the promoter and 3' flanking region of the gene. However, each contains the BLG promoter, the EcoRV site and the BLG 3'-flanking sequence. For each recombinant, the 1.5 kbp KpnI fragment from pWAP FIB is isolated, blunt ends generated as above, and the product  
15 ligated to EcoRV-cleaved vector sequences. Those constructs with the proper structures, determined as described above, can be used to express FIB in the biological fluids of transgenic animals.

20 A notable result is obtained with doubly-transgenic mice that are produced having native BLG genomic sequences which were injected as separate constructs to be concatenated *in vivo*, as additional flanking sequences to the BLG target cDNA construct (such as, BLG promoter-Intron I-EcoRV-Intron VI-BLG 3' flank plus BLG).  
25 In particular, such mice give higher expression more frequently than do mice <sup>Produced</sup> ~~produced~~ using constructs of the BLG promoter-FIB cDNA-BLG gene or BLG promoter-FIB genomic ( $\pm$  BLG 3' end). Intact or native BLG genomic sequences that are preligated to the BLG-cDNA target may  
30 give the same advantage. The same principle applies, *mutatis mutandis*, to WAP genomic sequences.

#### Isolation of Milk From Lactating Transgenic Animals

Obtaining milk from a transgenic animal according to the present invention is accomplished by conventional  
35 means. See, for example, McBurney et al., *J. Lab. Clin. Med.* 64: 485 (1964); Velandar et al., *Proc. Nat'l Acad.*

Sci. USA 89: 12003 (1992), the respective contents of which are incorporated by reference. Fibrinogen or subunit chains or protein products thereof can be isolated and purified from milk or urine by conventional means without deleteriously affecting activity. A highly preferred method entails a combination of anion exchange and immunochromatographies, cryoprecipitations, and zinc ion-induced precipitation of either whole milk or milk whey (defatted milk) proteins. For these techniques, see Bringe et al., *J. Dairy Res.* 56:543 (1989), the contents of which is incorporated herein by reference.

Milk is known to contain a number of proteases that have the potential to degrade foreign proteins. These include in the main an alkaline protease with tryptic and chymotryptic activities, a serine protease, a chymotrypsin-like enzyme, an aminopeptidase and an acid protease. Clark et al. (1987), *supra*. It may be desirable, therefore, to protect newly secreted FIB or subunit chains thereof against proteolytic degradation. Such precautions include rapid processing of the milk after collection and addition to the milk of well known inhibitors of proteolysis, such as are listed in SIGMA CHEMICAL CO. CATALOG, 1993 edition, at page 850.

#### Detection and Quantification of Transgenic FIB

The present inventors have used two sandwich ELISA formats to detect and quantify FIB in milk whey. One technique is a polyclonal antibody capture/FIB/monoclonal antibody detection system ("P/M") and the other which uses polyclonal antibodies both for capture and for detection ("P/P"). Both methods are described below. The P/M system benefits from greater specificity for hFIB as it recognizes only one epitope of about 6 to 20 amino acids in length specific to hFIB. However, because the P/M system recognizes only a single epitope, detection sensitivity is reduced. In contrast, the P/P system will provide less specificity as it recognizes many epitopes,

but at the same time will provide greater detection sensitivity. As these multiple epitopes include epitopes conserved between mouse and human FIB, the background "FIB" signal is increased when measuring rhFIB in mouse milk by the P/P system. Detailed sandwich ELISA procedures are provided in EXAMPLE 7 below.

Purification and Biological Activity of Secreted Recombinant Fibrinogen

Mammalian proteins associated with hemostasis are well-conserved with respect to structure and function and thus it is necessary to both identify and purify rFIB from host body fluids. To establish that the FIB secreted in the milk of transgenic animals is indeed authentic, i.e., physiologically active, heterologous FIB, a number of well known, standard techniques may be carried out; these are listed below:

*Physical techniques*

(1) Physical separation and identification. A sequence of ion exchange chromatography and  $Zn^{2+}$  selective precipitation achieves two main goals: the reduction of background protein (i.e., host FIB) from milk and an efficient isolation of rhFIB away from contaminating host FIB. Host FIB is present to a very small extent from natural passage of serum proteins into milk, but it is predominately due to injury of the mammary tissue from the milk collection process. Over half of the background milk proteins can be removed by anion exchange chromatography. Over 75% of the casein will be removed by the  $Zn^{2+}$  selective precipitation process. The resulting rhFIB will be about 20-30% pure at this stage with an overall yield of about 50%. Control host milk whey serves as a negative human FIB control, and control whey doped with authentic human FIB serves as a positive control for human FIB. Transgenic whey serves as the source of recombinant FIB. The non-doped control whey contains host FIB that primarily

partitions into a second 24 mM  $Zn^{2+}$  supernatant fluid. Only about 1-3% of the FIB present in the final purified human or rhFIB samples will be host FIB. Thus, the  $Zn^{2+}$  selective procedure enables an efficient separation of rhFIB from host FIB.

(2) Silver staining of SDS-PAGE gels of isolated rhFIB.

(3) Western blot analyses of isolated rhFIB.

(4) Fibrinopeptide A detection by EIA in thrombin-treated samples.

#### *Biochemical tests*

(5) Using ELISA signals (using detection by polyclonal anti-human fibrinogen antisera found in the supernatants after centrifugation to remove precipitates and/or clots) to track the consumption of isolated rhFIB by human thrombin.

(6) Determination of the apparent molecular weights of the assembled FIB molecule and of the separate  $\alpha$ ,  $\beta$ , and  $\gamma$  chains found under reducing conditions.

(7) The formation of a fibrin-like clot after treatment of the isolated rhFIB with human thrombin.

(8) The formation of  $\alpha$ ,  $\beta$ , and  $\gamma$  fibrin chains in these same clots.

(9) The release of fibrinopeptide A to confirm biological activity of isolated rhFIB by conversion to fibrin upon reaction with human thrombin.

For sources of these well-known techniques see, for example, Hartwig et al., *J. Biol. Chem.*, 266: 6578 (1991); Koopman et al., *J. Biol. Chem.*, 266: 13456 (1991); Lonnerdal et al., *J. Appl. Biochem.*, 4: 203 (1982); Farrell et al., *Biochem.*, 30: 9414 (1991); Goding, *MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE*, Academic Press, NY; Bolyard et al., *Gene* 66: 183 (1988); Hartwig et al., *J. Biol. Chem.*, 266: 6578 (1991); Roy et al., *loc. cit.*, 266: 4758 (1991); Dempfie et al., *Thrombosis Res.*, 46: 19 (1987); Dempfie et al., *loc. cit.*, 48: 223 (1987); and Yu et al., *Biochem. Biophys.*

*Res. Commun.*, 96: 1032 (1980), the respective contents of which are incorporated by reference.

The present invention is further described by reference to the following illustrative examples.

5

#### EXAMPLE 1

#### WAP-Fibrinogen cDNA Constructs For Expression of Fibrinogen in Transgenic Animals

##### Construction of cassette vectors

10 FIB subunit chain DNAs, tissue-specific promoters, and secretion signal sequences were obtained from sources described above. FIB subunit chain cDNAs were cloned into a modified pUC 18 vector, and grown up in *E. coli* JM109.

15 A pUC18 vector (GIBCO-BRL, Gaithersburg, MD) was digested with HindIII + EcoRI restriction endonucleases, blunted with T4 DNA polymerase in the presence of 100 mM dNTPs, and a Not I linker was ligated into the former HindIII-EcoRI multiple cloning site. This modified pUC fragment was additionally digested with Not I+ enzyme to remove extra (multiple copies) NotI linker sequences  
20 arising from ligation, and then religated and grown up in *E. coli* JM 109. This procedure modified the pUC18 vector by removing the entire multiple cloning region of pUC18 (including the Kpn I site) and replacing it with a Not I restriction site. The new vector was designated  
25 pUCNot1+.

A pUC vector containing ~2.6 kbp of WAP 5' promoter region, ~1.3 kbp of WAP 3' UTR and flanking 3', but no WAP coding or intronic regions was constructed (designated cassette vector pUCWAP4, Figure 2; see WAP  
30 gene, Figure 1, and cassette vector pUCWAP5, Figure 3). The entire murine WAP gene including 2.6 kb of 5' untranslated sequence and 3' untranslated region may be obtained from Dr. I. Hennighausen, National Institutes of Health, Bethesda, Maryland. See, Campbell et al.,  
35 *Nucleic Acids Res.*, 12:8685 (1984). The WAP fragment contained within the WAP4 vector contains a ~2.6 kbp WAP

promoter 5' region beginning at EcoRI#1 and ending at the translational start site of WAP which is immediately downstream of the unique KpnI endonuclease restriction site (see Figure 1). To this KpnI site was ligated a  
5 fragment of ~1.3 kbp of WAP UTR and flanking 3' sequence. This WAP 3' DNA included the region from immediately downstream of the WAP stop codon down to the EcoRI#2 site. The WAP fragment contained in WAP4 was excised from the pUC vector using EcoRI, and then blunted, and  
10 NotI linkers were added, further trimmed by NotI digestion, and ligated into the pUCNotI+ plasmid which had been linearized with NotI restriction endonuclease. The resulting plasmid was designated pUCWAP5 (see Figure 3).

15 Amplification by PCR of fibrinogen subunit chain cDNAs

Each of the cDNAs for A $\alpha$ , B $\beta$ , and G $\gamma$  chains for human fibrinogen were individually modified and amplified by polymerase chain reaction (PCR) to create KpnI endonuclease restriction sites on their 5' and 3' ends.  
20 The 5' KpnI site was engineered by PCR using the primers [containing the 6 base sequence (GGTACC shown underlined in Table 1)] that immediately flanks the ATG start codon in the cDNAs of A $\alpha$ , B $\beta$ , and G $\gamma$  chains for hFIB. After amplification, the ends of the extension using PCR  
25 products were blunted by T4-polymerase (in the presence of deoxynucleosidetriphosphates to inhibit processive exonuclease activity. In a similar fashion, a KpnI site was engineered by site modification into the 6 base sequence GGTACC shown underlined in Table 1 immediately  
30 flanking the stop sequence in the 3' UTR of each cDNA for A $\alpha$ , B $\beta$ , and G $\gamma$  chains for hFIB. The complement of the stop sequences is shown in bold in the 3' primers in Table 1.

Table 1

Oligonucleotides for Amplifying FIB Subunit Chain cDNA

Seq. ID No. 3

A $\alpha$  5' GCTAGGTACCATGTTTTCATGAGGATCGT

5 Seq. ID No. 4

A $\alpha$  3' CAGTGGTACCCTAGACAGGGCGAGATTTAG

Seq. ID No. 5

BB 5' GCTAGGTACCATGAAAAGAATGGTTTCGTG

Seq. ID No. 6

10 BB 3' CAGTGGTACCCTATTGCTGTGGGAAGAAGG

Seq. ID No. 7

G $\gamma$  5' GCTAGGTACCATGAGTTGGTCCTTGACCCC

Seq. ID No. 8

G $\gamma$  3' CAGTGGTACCTTAAACGTCTCCAGCCTGTT

15 GGTACC = KpnI site

ATG = start codon

CTA and TTA = stop codon

Construction of pUC plasmids containing  
fibrinogen subunit chain cDNA

20 The blunted PCR products of the cDNAs for the B $\beta$  and  
G $\gamma$  chains of human fibrinogen were digested with KpnI  
restriction endonuclease. In the case of the A $\alpha$  chain,  
the PCR product was blunt-end cloned into pUCNotI+ (which  
had been digested with NotI and blunted with T4  
25 Polymerase) prior to partial KpnI digestion. This  
intermediate cloning and partial digestion step was  
necessary to generate intact coding fragment due to the  
presence of an internal KpnI site within the A $\alpha$  chain  
cDNA. The intact A $\alpha$  chain cDNA fragment was selected by  
30 gel electrophoresis, and cloned into the KpnI site at the  
junction between the WAP 5' promoter and WAP 3'  
UTR/flanking sequences within the pUCWAP5 plasmid. The  
KpnI-digested PCR products from B $\beta$  and G $\gamma$  chains for  
human fibrinogen were each directly cloned into a pUCWAP5  
35 plasmid at the KpnI site. Separate electroporation  
transformation reactions were done on *E.coli* using each



of the three pUCWAP5/fibrinogen cDNA preparations, and colonies were picked and grown up in TB ampicillin broth. Plasmid preparations from these colonies were analyzed by restriction enzyme digestion and gel electrophoresis.

5 The correct size and orientations were selected and one clone for each WAP-fibrinogen cDNA construct was sequenced at the WAP promoter 5':fibrinogen cDNA and fibrinogen cDNA:WAP 3' UTR and flanking junctions. Schematics summarizing the construction of the WAP- $\alpha$  (about 5.8 kbp), - $\beta$  (about 5.4 kbp), and - $\gamma$  (about 5.2 kbp) cDNA plasmid and linearized transgenes for human fibrinogen are given in Figures 4, 5, and 6, respectively.

15

#### EXAMPLE 2

##### Preparation of DNAs for Microinjection

The intact and linearized WAP 5' promoter/fibrinogen cDNA/WAP 3' UTR and flanking fragments were excised from each pUCWAP5 plasmids by NotI restriction endonuclease digestion and purified by low melting point agarose electrophoresis. The DNA:agarose band was cut from the gel slab. The agarose band was then treated with agarase to degrade and remove agarose contamination

25 After digestion, the solution containing the cDNA was brought to 10 mM  $Mg^{2+}$ , 20 mM EDTA and 0.1% SDS and then extracted with phenol/chloroform. DNA was precipitated from the aqueous layer with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate at  $-20^{\circ}C$  overnight. After centrifugation, the pellet was washed with 70% ethanol, dried, and each of the constructs was resuspended and dissolved in Brinster microinjection buffer to a total ( $\alpha$ ,  $\beta$ , plus  $\gamma$ ) concentration of about 1.2 to 5  $\mu g/ml$ . Brinster et al., *Proc Natl. Acad. Sci. USA* 82:4438 (1985), incorporated by reference.

EXAMPLE 3

Transgenic Mice

Transgenic mice were produced essentially as described by Hogan et al., *Manipulating The Mouse Embryo*, Cold Spring Harbor Press, 1986, incorporated by reference. The procedures employed are outlined below.

Glass needles for micro-injection were prepared using a micropipet puller and microforge. Injections were performed using a Nikon microscope having Hoffman Modulation Contrast optics, with Narashigi micromanipulators and a pico-injector driven by N<sub>2</sub> (Narashigi).

Fertilized mouse embryos were surgically removed from the oviducts of superovulated female CD-1 mice and placed into M2 medium. Cumulus cells were removed from the embryos with hyaluronidase at 300 µg/ml. The embryos were then rinsed in new M2 medium, and transferred into M16 medium for storage at 37° C prior to injection.

Stock solutions containing about 1.2 µg/ml (having about 0.4 µg/ml each of the linearized constructs containing the A $\alpha$ , B $\beta$ , and G $\gamma$  chains cDNAs for human fibrinogen, or about 60-70 copies/pl of each construct) were prepared and microinjected into the pronuclei of 1 cell mouse embryos. In addition, stock solutions containing about 5 µg/ml total DNA (having about 1.7 µg/ml of linearized constructs containing each of the A $\alpha$ , B $\beta$ , and G $\gamma$  chains cDNAs for human fibrinogen or about 200 copies/pl of each construct) were prepared and microinjected into the pronuclei of 1 cell mouse embryos.

After injecting the DNA solution into the male pronucleus, embryos were implanted into avertin-anesthetized CD-1 recipient females made pseudo-pregnant by mating with vasectomized males. About 25-30 microinjected mouse embryos per recipient were transferred into pseudopregnant females. Embryos were allowed to come to term and the newborn mice were

analyzed for the presence of the transgene by PCR using murine WAP and a FIB-specific primer as described below.

Table 2 summarizes the embryo transfer data from an experiment that produced transgenic fibrinogen-producing mice. Separate PCR analysis for detection of each of the cDNAs of fibrinogen was done on DNA purified from biopsied tail tissues by the method shown in Example 4 below. The oligonucleotide primers shown in Table 3 were used to detect each of the FIB cDNAs. Detection of each chain required a WAP oligonucleotide (no. 1 in Table 3) and one of the other oligonucleotides (Nos. 2, 3 or 4).

Table 2

Transfers of Fibrinogen Constructs

Thirty transfers with the FIB constructs were performed. The three subunit chain constructs were co-injected at total DNA concentrations of 1.2  $\mu\text{g/ml}$  and 5  $\mu\text{g/ml}$  or 200 total copies/pl and 800 total copies/pl, respectively. Fourteen transfers were made with 1.2  $\mu\text{g/ml}$ -injected embryos and sixteen with 5  $\mu\text{g/ml}$ -inject embryos. Eighteen Founder ("Fo") animals were produced that carried at least one construct. Eight Fo mice were mated with control mice which resulted in five lines transmitting the transgene. First generation offspring ("F1") from lines 1, 23 and 85 have been bred and litters produced.

	1.2 $\mu\text{g/ml}$	5 $\mu\text{g/ml}$
Transfers:	14	16
Pups alive:	68	69
Embryos injected:	696	1098
Embryos transferred:	480	675
Embryos per transferred:	34.2	42.1
Pups per litter	6.2	5.3
Transfers pregnant:	11	13
Pregnancy rate:	78%	81%

Mice tested:	68	69
Number transgenic:	7	11
Percent transgenic:	10%	15%
% Positive for $\alpha, \beta, \gamma$ :	85%	72%
5 $\alpha, \beta, \gamma$ Transgenic females:	3	3

Table 3

**Oligonucleotide Primers Used for PCR Detection of Human Fibrinogen Transgenic Mouse**

T0290

10	Seq. ID No. 9.	Sense strand.	WAP-specific oligonucleotides.
			5'-CTGTGTGGCCAAGAAGGAAGTGTTG-3'
	Seq. ID No. 10.	Antisense oligonucleotide.	A $\alpha$ chain detection.
			5'-GATGTCTTTCCACAACCCTTGGGC-3'
15	Seq. ID No. 11.	Antisense oligonucleotide.	B $\beta$ chain detection.
			5'-CCCGATAGCCACCTCCTCTGATG-3'
	Seq. ID No. 12.	Antisense oligonucleotide.	G $\gamma$ chain detection.
20			5'-CCTGGACTTCAAAGTAGCAGCGTC-3'

25 A summary of the types of fibrinogen transgenic mice detected in founder and offspring of founder mice is given in Table 4. A listing of the positive FIB triple construct founders (Fo) and a listing of  $\beta$ -chain positive transgenic mice are also given in Table 4.

Table 4

**Transmission of Fibrinogen Transgenes**

T0291

Mouse No.	Transmission Frequency	Genes Transmitted
30 1 ( $\alpha, \beta, \gamma$ )	9/13 (69%)	$\alpha, \beta, \gamma$
23 ( $\alpha, \beta, \gamma$ )	6/12 (50%)	$\alpha, \beta, \gamma$ (3/6); $\beta$ only 3/6)
109 ( $\alpha, \beta, \gamma$ )	5/11 (45%)	$\alpha, \beta, \gamma$

	112 ( $\alpha, \beta, \gamma$ )	5/8 (62%)	$\alpha, \beta, \gamma$ (4/6) $\alpha$ only (1/6) $\alpha, \gamma$ only (1/6)
	85 ( $\alpha, \beta$ )	1/6 (16%)	$\beta$ only
5	113 ( $\alpha\beta\gamma$ )	0/8	None
	89 ( $\alpha$ )	0/6	None

Positive Fibrinogen Triple Construct F1's

1-2, 1-5, 1-6, 1-11, 1-12, 1-14, 1-16, 1-17, 1-20, 23-3, 23-4, 23-12, 109-3, 109-4, 109-5, 109-10, 109-11, 112-3, 112-6, 112-7, 112-8

$\beta$ -Chain Positive Mice

23-2, 23-7, 23-11, 85-2

All embryos were injected with the three FIB constructs at a total DNA concentration of 5  $\mu\text{g/ml}$  or about 200-300 copies of each construct per pl.

A family tree of transgene passage from Fo to F1 females is shown Figure 7. Males were not retained.

EXAMPLE 4

Transgenic Animal DNA

DNA can be prepared from tissue of a transgenic animal of any species by the method exemplified below for mice. Marmur, *J. Mol. Biol.* 3:208 (1961), incorporated by reference.

A 5 mm piece of mouse tail was removed from young, potentially transgenic mice at weaning (3 weeks) age, and frozen in liquid nitrogen. To the frozen tissue was added 840  $\mu\text{l}$  of Lysing Solution (8 mM EDTA-0.8% SDS-0.8% 2-mercaptoethanol-80  $\mu\text{g/ml}$  Proteinase K-1 M sodium chlorate in 40 mM TRIS buffer pH 8.0 and 120 mM NaCl, and the mixture incubated at 50°C. The mixture was then extracted with 250  $\mu\text{l}$  of phenol/chloroform/isoamyl alcohol (25:24:1) for 10-15 seconds, then centrifuged for 10 minutes. The supernatant fluid (about 830  $\mu\text{l}$ ) was removed to a fresh tube, and a DNA clot produced by vortexing the solution with 0.6 vols. of isopropanol. The mother liquor

was decanted, and the DNA clot rinsed twice with 80% ethanol. The DNA clot was isolated by 5 minutes of centrifugation, aspiration of the supernatant fluid, and air drying of the clot with a stream of air for 10 minutes.

The DNA clot was dissolved in 250  $\mu$ l of TE buffer (10 mM Tris.HCl, pH 7.0-1mM EDTA, and the solution treated with 10  $\mu$ l of a RNase mixture (1 mg/ml RNase A and 4,000 units/ml RNase T1) for 1 h at 37°C. This mixture was shaken with 50  $\mu$ l of a 24:1 (v/v) solution of chloroform-isoamyl alcohol for 5-10 secs., centrifuged, and the supernatant fluid transferred to a fresh tube.

The recovered supernatant fluid above was mixed sequentially with 25  $\mu$ l of 3 M sodium acetate and 0.5 ml of 95% ethanol. The supernatant fluid was decanted from the precipitated DNA, and the precipitate washed with 80% ethanol. The purified DNA was isolated by centrifugation, air dried, then dissolved in 150  $\mu$ l of TE.

Essentially the same technique was used to prepare DNA from pigs, and the same or similar techniques can be used to prepare DNA from other animals.

PCR reactions on DNA samples isolated from transgenic animal tissue samples were carried out conventionally, as described above. Concentrations of DNA isolates were determined by measuring the absorbency at 260 nm of 1:20 dilutions of the TE solutions described above. A portion of the sample was then diluted into 100  $\mu$ l of TE to a concentration of 30 ng/ $\mu$ l.

PCR reaction mixtures (20  $\mu$ l volume)

	Component	Volume ( $\mu$ l)	Concentration
	HOH	13.25	-
	10X Taq buffer	2.5	1
5	dNTPs	2.0	0.2 mM
	MgCl <sub>2</sub>	1.5	1.5 mM
	Primer 1	0.3125	0.5 $\mu$ M
	Primer 2	0.3125	0.5 $\mu$ M
	Taq polymerase	0.125	25 $\mu$ units/ $\mu$ l
10	DNA template	1.0	1.2 ng/ $\mu$ l

Procedure

Embryo lysing buffer (5  $\mu$ l, 0.9% Nonidet P-40 and 0.9% Tween 20 in 20 mM TRIS buffer) was added to each PCR tube along with 1  $\mu$ l of prepared DNA solution, and the mixture overlaid with 25  $\mu$ l of mineral oil. The tubes were placed in an automatic temperature cycler (such as that manufactured by N.J. Research) and heated to 98°C for 2 minutes; the temperature was then brought down to 85°C.

PCR reaction mixture (20  $\mu$ l) was added to each tube and 40 cycles of the following protocol carried out:

Denaturation at 96°C for 20 seconds

Annealing at 55°C for 1 minute

Elongation at 75°C for 30 seconds

PCR reaction products were analyzed by running 20% of the reaction mixture products on agarose gels and identifying fragment sizes by comparison with marker DNA fragments.

Figure 7 shows that a variety of founder animals having A+ and/or B+ and/or G+ constructs can transmit all three or some of the A+, B+, and G+ transgenes. All animals were produced from embryos having coinjections of the same DNA solution containing all of the A+, B+, and G+ constructs. Detection of A+, B+ and G+ DNAs was done by PCR as described above. Founder animals 1, 23, 109 and 112 had all three transgenes. Founder animal 85 had only A+ and B+. These founder animals were outbred with

control (nontransgenic) mice. Animals 1 and 109 passed all three transgenes to 5 of 13 and 5 of 11 offspring, respectively, which is typical of Mendelian genomic transmission for a single allele. Founder animals 23 and 112 transmitted some transgene to 6 of 12 and 7 of 8 animals, respectively. Several offspring of animal 23 had only B+, while one offspring of animal 112 had only A+ and another had A+ and G+. Thus, founder animals 23 and 112 are examples of triple transgenic animals having a confirmed separate B+ allele in the case of animal 23 and having a separate A+ and B+ allele in the case of animal 112. Founder animal 85 is also an example of a mouse with a B+ only allele. Thus, crossbreeding of animals having separate alleles and therefore loci for each gene can be done in order to increase expression (the control over a rate limited B+ chain may be overcome by having a B+ allele with a more inducible loci). Furthermore, double and triple heterozygotes of the same gene (either A+ and/or B+ and/or G+) can be achieved to boost production levels (i.e., produce a A+ B+ G+, B+ animal by crossbreeding a B+ animal, and further crossbreeding that A+B+G+,B+ animal with a different B+ line to obtain A+ B+ G+,B+ B+).

#### EXAMPLE 5

##### Transgenic Pigs

Embryos were recovered from the oviduct. They were placed into a 1.5 ml microfuge tube containing approximately 0.5 ml embryo transfer media (phosphate buffered saline + 10% fetal calf serum, Gibco BRL). These were then centrifuged for 12 minutes at 16,000 x g RCF (13,450 RPM) in a microcentrifuge (Allied Instruments, model 235C). Embryos were removed from the microfuge tube with a drawn and polished Pasteur pipette and placed into a 35 mm petri dish for examination. If the cytoplasm was still opaque with lipid such that pronuclei were not visible, the embryos were centrifuged



again for 15 minutes. Embryos to be microinjected were placed into a microdrop of media (approximately 100  $\mu$ l) in the center of the lid of a 100 mm petri dish. Silicone oil was used to cover the microdrop and fill the lid to prevent media from evaporating. The petri dish lid containing the embryos was set onto an inverted microscope (Carl Zeiss) equipped with both a heated stage and Hoffman Modulation Contrast optics (200 x final magnification). A finely drawn (Kopf Vertical Pipette Puller, model 720) and polished (Narishige microforge, model MF-35) micropipette was used to stabilize the embryos while about 1 - 2 picoliters of HPLC-purified DNA solution containing approximately 200-500 copies of DNA construct was delivered into the male pronucleus with another finely drawn micropipette. Embryos surviving the microinjection process as judged by morphological observation were loaded into a polypropylene tube (2mm ID) for transfer into the recipient pig. Seven surgical transfers of pig embryos were made. These embryos had been coinjected with the WAP-fibrinogen cDNA constructs used in the above mentioned transgenic fibrinogen mice. Only one of these recipient gilts was confirmed pregnant by ultrasound. The (apparent) non-pregnant animals were kept to tentative term as their (potential) litter sizes may have been too small to detect by ultrasound analysis (In the past, three litters of four piglets or fewer were not detected by ultrasound. Some of these litters produced transgenic animals.)

Table 5

Fibrinogen Porcine Embryo Data

	<u>Expt. 1</u>	<u>Expt. 2</u>	Transfer Totals 10/25/93 ID
5			
	Number of donors: 8	8	Transfer#1 50 embryos #129
10	Number of ovulations: 261	283	Transfer#2 47 embryos #147
	Embryos collected: 250	296	Transfer#3 44 embryos #108
	Number of UFO 's: 99	77	
	UFO's that cleaved: 29	6	
15	Number injected: 141	219	10/28
	Number transferred: 141	200	Transfer#1 50 embryos #114
	Transfers: 3	4	Transfer#2 50 embryos #139
20	Embryos/Transfer: 47	50	Transfer#3 50 embryos #140
	Ovulations/Pig: 32.6	35.3	Transfer#4 50 embryos #131
25	Embryos/Pig: 31.2	37	

All embryos were injected with the three fibrinogen constructs at a total DNA concentration of 5 µg/ml or about 200-300 copies of each construct per pl.

EXAMPLE 6

30 Preparation of Milk and Whey from Transgenic Animals

Lactating mice were milked once on about day 12 of lactation. The mice were first separated from their young for approximately 5 hours. They were then anesthetized with 0.4 ml avertin at 2.5% (im.), and 0.2 ml oxytocin was then administered at 2.5 IU/ml (ip.) to

permit release of the milk. A milking device consisting of a vacuum pump (2.5 psi) and syringe with an Eppendorf tip was used to direct milk into an eppendorf tube. As noted above, milk should not be placed on ice because of cryoprecipitation of fibrinogen.

To prepare whey, milk was diluted 1:1 with TRIS buffer (0.03 M Tris pH 7.4; 0.06 M NaCl) and centrifuged in a TLA-100 rotor in a Beckman TL-100 table top ultracentrifuge at 51,000 rpm (89,000 x g) for 30 minutes at room temperature. After centrifugation, the whey (infranatant fluid) was collected with an 18 gauge needle, leaving the casein pellet and upper cream layer in the tube. To remove solids or cream that co-transferred during the initial recovery, the whey obtained from the first centrifugation was subjected to a second spin at 12,000 rpm for 30 minutes in a TMA-4 rotor in a Tomy MTX-150 centrifuge. Following the second spin, the whey was recovered as before.

#### EXAMPLE 7

##### Determination of Fibrinogen in Milk by a Sandwich ELISA

A sandwich ELISA was used to measure the amount of FIB protein produced by founder and progenic transgenic animals in the diluted skim or defatted milk (here termed "whey"); the whey value is used to estimate the concentration of FIB in whole milk.

Generally, in the first step of the P/M ELISA system, a fibrinogen or fibrinogen chain analyte-containing sample (for example, whey) is incubated with a mouse anti-human fibrinogen mAb (clonal number 311, American Diagnostics) that is reactive with the alpha chain of fibrinogen to form a mAb-fibrinogen (or chain) first complex. This first complex is then "captured" by incubation with rabbit polyclonal anti-human fibrinogen antibodies immobilized in microliter plate wells. In the detection step, the captured fibrinogen (or alpha chains) is contacted with labeled (horse radish peroxidase) anti-

mouse IgG, and the amount of label quantified by reaction with o-phenylenediamine and measurement of absorbency at 490 nm; this is a measure of the amount of analyte present. Standard curves were obtained by spiking the whey of control (nontransgenic) animals with pure reference human FIB material. Thus, the presence of background amounts of FIB is normalized by the standard curve. This sandwich ELISA gives very low background values (about 0.06 absorbency units at 490 nm) for mouse whey alone with no human FIB. Note the typical signals given in Table 6 are about 0.25 or higher absorbency units at this wavelength.

In the P/P system, Immulon II 96-well microliter plates were coated with 100  $\mu$ l/well of 1:500 diluted rabbit anti-human FIB (Celsus cat. no. 60960) in 0.1 M  $\text{NaHCO}_3$ -0.1 M NaCl, pH 9.6, for 24 hours at refrigerator temperatures. Coated wells were washed three times with Wash Buffer (25 mM Tris-HCl-50 mM NaCl-0.5% Tween-20, pH 7.0) and blocked for 20 minutes with Dilution Buffer (1 mg/ml PEG in 25 mM Tris-HCl-50 mM NaCl, pH 7.0) at room temperature. A volume of 100  $\mu$ l of standard human FIB (100 ng/ml to 1.9 ng/ml range) and/or 100  $\mu$ l of diluted milk sample was added to each well, and the mixtures incubated at 37°C for 45 minutes. The wells were washed and blocked as above for 10 minutes at room temperature. 100  $\mu$ l of 1:1000 diluted horse radish peroxide-conjugated rabbit anti-human FIB were added to each well, and the mixtures incubated at 37°C for 45 minutes. After washing the wells as above, 100  $\mu$ l of o-phenylenediamine solution (1 tablet/5 ml diluent) were added to each well. After waiting about 2-3 minutes for color development, reactions were stopped by the addition to each well of 100  $\mu$ l of 3 N  $\text{H}_2\text{SO}_4$ . Plates were then read at 490 nm.

Table 6 presents the results of the analyses of rhFIB by the P/M ELISA system described above in the first lactation whey (and in whole milk by calculation) of three founder (Fo) transgenic mice whose genome

contained each of the A $\alpha$ , B $\beta$ , and G $\gamma$  chains cDNAs for human fibrinogen. In this experiment, "whey" refers to skim milk diluted 1.7X with EDTA to solubilize the caseins. The averages of four dilutions of each whey sample for the three test animals was about 16.3, 3.86 and 7.55  $\mu$ g/ml, respectively. The corresponding values for whole milk were 27.6, 6.56 and 12.8. Second lactation production of rhFIB was similar.

In another experiment with the same animals the first lactation whey fibrinogen concentrations averaged about  $22 \pm 4$  (animal 1),  $9 \pm 2$  (animal 22) and  $11 \pm 3$  (animal 23)  $\mu$ g/ml. The second lactation of animal 1 produced  $17 \pm 3$   $\mu$ g/ml whey.

The P/M ELISA system was used to assay for rhFIB in the third lactation milk of mouse no. 1/Fo and the first lactation of mouse no. 1 female offspring (1-11/F1). As shown in the data of Table 7, mouse no. 1/Fo had about 71  $\mu$ g/ml, and her offspring (no. 1-11/F1) had about 53  $\mu$ g/ml total FIB signal. Thus, the amount of rFIB after subtracting background is calculated to be about 50  $\mu$ g/ml for mouse no. 1/Fo and about 32  $\mu$ g/ml for offspring 1-11/F1, respectively, over that of endogenous FIB present from serum to milk passover. The slightly lower value (28  $\mu$ g/ml) of the P/M assay of the previous lactation of mouse no. 1/Fo is not significantly different than the P/P value when lower detection sensitivity of the former method is taken into account. It is noted that collection of mouse milk is inherently traumatic and leads to serum passover of endogenous mFIB.

In summary, mouse no. 1/Fo (founder) produced rFIB in milk at a level of about 30  $\mu$ g/ml or greater over 3 lactations as detected by either the P/M or P/P ELISA methods, and has passed this trait on into the F1 generation which, in mouse no. 1-11/F1, produced 32  $\mu$ g/ml rFIB.

TABLE 7

Inheritability of Transgenic rhFIB Gene

Mouse No.   μg rhFIB/ml 5X   μg rhFIB/ml milk

diluted whey

5	1/Fo	14.0 ± 2.0	71.0
	1-11/F1	10.4 ± 0.8	53.0
	Control whey	4.0 ± 0.7	21.0

10      These productions are substantially greater than the  
2 μg/ml produced in the COS1 system described by Roy et  
al., 1991, above.

15      Western analysis of control mouse milk using  
polyclonal anti-human fibrinogen antibody showed about 21  
μg/ml of background fibrinogen signal (see Table 7).  
Western analysis that uses the clonal number 311 or Y18  
monoclonal antibody has a lower limit of sensitivity of  
about 1 μl sample of 100 μg/ml fibrinogen (Phastgel  
system) or 20 μl of 25 μg/ml in a 3" by 4" SDS-PAGE gel.

To 400

TABLE 6

	A	B	C	D	E	WHEY	MILK
1	Animal	Dilutions	AVG ABS 490nm		FIB $\mu\text{g/ml}$	AVG FIB $\mu\text{g/ml}$	AVG FIB $\mu\text{g/ml}$
2	#1 DAY 6	100	0.623	0.132	13.2		
3		200	0.523	0.088	17.6	16.3	27.6
4		400	0.434	0.049	19.4		
5		800	0.334	0.018	14.7		
6							
7	#22	100	0.374	0.022	2.16		
8		200	0.335	0.018	3.69	3.86	6.56
9		400	0.273	0.014	5.73		
10		800	0.251	0.013	10.3		
11							
12	#23	100	0.477	0.067	6.75		
13		200	0.384	0.026	5.22	7.55	12.8
14		400	0.338	0.019	7.44		
15		800	0.261	0.013	10.8		

5

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15

45

EXAMPLE 8

**Confirmation That Transgenic Fibrinogen is Actually  
Recombinant Human Fibrinogen**

**Materials Used to Analyze Fibrinogen**

5 Rabbit antisera against human fibrinogen was  
purchased from Celsus laboratories (Cincinnati, OH.).  
Rabbit antisera against human fibrinogen conjugated to  
horseradish peroxidase (HRP) was purchased from Dako  
corporation (Carpenteria, CA.). Anti-rabbit  
10 immunoglobulins conjugated to HRP were purchased from  
Sigma Chemical Company (St. Louis, MO). Synthetic human  
fibrinopeptide A was purchased from Sigma Chemical  
Company. Rabbit antisera against human fibrinopeptide A  
was purchased from Nordic Immunological Laboratories  
15 (Capistrano Beach, CA.). Immulon II microtiter plates  
were purchased from Fisher Scientific (Pittsburgh, PA.).  
Plasma derived human fibrinogen was provided by American  
Red Cross. (Rockville, MD.). O-phenylenediamine-2HCl  
(OPD) tablets were purchased from Abbott Laboratories  
20 (Chicago, IL.). Metal enhanced DAB substrate was  
purchased from Pierce Chemical Company (Rockford, IL.).  
Centricon diafilters were purchased from Amicon (Bedford,  
MA.). 4-15% gradient phast-gels were purchased from  
Pharmacia Biotech (Piscataway, NJ.). Nitrocellulose  
25 papers (40 microns pore size) were purchased from Sigma  
Chemical Company. DEAE-Fast flow sepharose was purchased  
from Pharmacia Biotech (Piscataway, NJ.). DEAE  
chromatography was performed with Pharmacia C-10 columns  
(15 cm x 1 cm), a Masterflex peristaltic pump, a Knauer  
30 spectrophotometer, and a Rainin data acquisition system  
were used to monitor chromatography. All other reagents  
were purchased from Sigma Chemical Company at the best  
grade available. Columns were kept at 4° C with a Lauda  
Super RMT water cooler.

35 A. **Separation of transgenic human FIB from host  
mouse FIB**

A sequence of anion exchange adsorption to reduce  
background protein content followed by a 2-step zinc ion-



selective separation of hFIB or rhFIB from mouse FIB was done on mouse milk whey and mouse plasma samples (Figure 8). Milk from transgenic and non-injected control mice were diluted with an equal volume of 200 mM EDTA, pH 7.0.

5 An aliquot of milk from control (nontransgenic mice) was spiked with known hFIB to give a final concentration of 30-50  $\mu\text{g/ml}$ , and diluted with 1 part EDTA. The milk:EDTA mixture was centrifuged at 4400 rpm for 45 minutes and the whey phase was separated from the top fat layer.

10 Mouse blood was used as a positive control to measure cross-reactivity of anti-human fibrinogen antisera to mouse fibrinogen. All samples were diafiltered against TBS (25 mM Tris-HCl, 100 mM NaCl, pH 7.0) and then were fractionated on a DEAE Fast Flow Sepharose packed column

15 at a linear velocity of 1 cm/min. The column dimensions were: 1 cm bed diameter and 6 cm in length. A fraction obtained by elution with 0.2 M NaCl fraction was diafiltered against TBS and reconstituted to the original sample volume. Using a 0.5 M  $\text{ZnCl}_2$  stock solution, the

20 zinc ion concentration in the samples was adjusted to 10 mM. This gave a visible precipitate which was separated by centrifugation at 10000 rpm (rotor diameter 6 inches) for 20 mins (pellet 1). Pellet-1 was dissolved in 100 mM EDTA, pH 7.0. Using the stock solution, the

25 zinc concentration in the supernatant fluid was adjusted to 24 mM. The resultant precipitate was centrifuged at 10000 rpm for 10 mins and the sediment (pellet-2) was dissolved in 100 mM EDTA, pH 7.0. EDTA solutions from

30 pellet-1 and pellet-2, as well as associated supernatant fluids, were analyzed for rhFIB by ELISA and the fractions were diafiltered against TBS.

Pellets from control mouse whey alone (no doped human fibrinogen) were dissolved to the same dilution factor as pellets from transgenic whey and human fibrinogen doped control whey. ELISA values for the dissolved transgenic whey and human fibrinogen doped control whey were 30-50  $\mu\text{g/ml}$  human fibrinogen. The control (pellet-2) when reconstituted to the same volume as TG (pellet-2) or hFIB

spiked control whey (pellet-2) gave a fibrinogen level of 1-2  $\mu\text{g/ml}$  as measured by ELISA.

The pellet-2 of non-transgenic whey was also reconstituted to a 20-fold more-concentrated level than transgenic whey and human fibrinogen doped control whey. This 20-fold concentrated, negative control was assayed by ELISA, and the value was then 30  $\mu\text{g/ml}$  human fibrinogen cross reactive equivalent signal with human fibrinogen antisera.

The dissolved pellets from transgenic whey and human fibrinogen doped control whey having 30-50  $\mu\text{g/ml}$  human fibrinogen by ELISA signal were applied to a phast-gel, SDS-PAGE (1  $\mu\text{l}$  sample volume applied to phastgel). A 1  $\mu\text{l}$  application of the 20 fold-more concentrated, negative-control, dissolved pellet also was applied to the gel.

B. Western blot analysis under non-reducing conditions

hFIB (reference) in assay buffer, diafiltered mouse plasma and rhFIB- rich fraction (pellet-2 of zinc separation step) of transgenic whey were mixed with non-reducing cocktail at 4:1 ratio and boiled for 5 minutes. Samples were analyzed on a 4-15% SDS-phast gel. Upon the completion of electrophoretic separation, the proteins were blotted onto a nitrocellulose membrane via "phast-gel diffusion blotting." The blot was probed for hFIB or rhFIB by a sandwich of rabbit anti-human fibrinogen antibody and anti-rabbit HRP conjugate antibody. Bound chromophore was visualized by metal enhanced DAB substrate. The results are shown in Figure 9.

Lanes 1 and 8 of Figure 9 show applications of hFIB reference protein at 100 and 10 ngs. Lanes 2 and 3 shows the transgenic pellet from mouse (1-11-8) at 15 ngs. Lanes 4 and show the transgenic pellet from mouse 1-6-8 at 30 ngs. Lane 6 shows mouse plasma derivative at 2-4  $\mu\text{g}$  of total protein application. Lane 8 shows pellet-2 from control whey.

The mobilities of rhFIBs under non-reducing conditions on 4-15% PAGE phast-gel from two different transgenic mice lines are similar to that of the plasma-derived hFIB reference (lanes 2-5). Intact mouse FIB from plasma (lane 6) and control whey pellet-2 (lane 7) migrated as multiple bands with a distinctly apparent higher molecular weight as compared to human FIB (lanes 1 and 10).

C. Western blot analysis under reducing conditions

hFIB (reference protein) in assay buffer, diafiltered mouse plasma and rhFIB-rich fraction (pellet-2 of zinc separation step) of transgenic whey were mixed with reducing cocktail at 4:1 ratio and boiled for 5 minutes. Samples were analyzed on a 8-25% SDS-phast gel. Upon the completion of electrophoretic separation, the proteins were blotted onto a nitrocellulose membrane via "phast-gel diffusion blotting." The blot was probed for hFIB or rhFIB by a sandwich of rabbit anti-human FIB and anti-rabbit HRP conjugate antibodies. Bound chromophore was visualized by metal-enhanced DAB substrate. The results are shown in Figure 10.

Lanes 1, 2 and 3 of Figure 10 show applications of hFIB reference at 100, 50 and 10 ngs. Lane 4 shows mouse plasma derivative (i.e. diafiltered 0.2 M NaCl eluate from DEAE FF Sepharose column). Lane 5, 6, 7 and 8 show applications of transgenic whey pellet-2 at 60, 30, 15 and 7.5 ngs.

Under reduced conditions,  $\alpha$ ,  $\beta$  and  $\gamma$  chains of reference hFIB from human plasma are clearly visible (lanes 1-3). Fibrinogen chains from mouse plasma (lane 4) have lower apparent molecular weights than human FIB, but different than rhFIB. Partially purified material from transgenic whey has three chains with lower apparent molecular weights than hFIB (lanes 5-8), but a higher molecular weight than mouse fibrinogen. The  $\alpha, \beta$  chains of rhFIB show up with greater intensity than the  $\gamma$  chain.

D. Thrombin assay

Diafiltered rhFIB-rich fractions from the zinc selective separation steps were suspended in 50 mM imidazole, 150 mM NaCl, 30 mM CaCl<sub>2</sub>, pH 8.0. Human thrombin was added to a final concentration of 4 U/ml and the samples were incubated at 37°C for 24 hours. Cloudy clot-like precipitates were clearly visible in TG (pellet-2), hFIB-spiked NTG (pellet-2), but not in control whey (pellet-2) after treated with thrombin. The clots were boiled for 3 mins and centrifuged at 10000 rpm for 10 minutes. The clot pellets were dissolved in reducing cocktail buffer. Assay supernatant was lyophilized and reconstituted to 1/10th the initial reaction volume. Supernatant was assayed for any residual fibrinogen and/or fibrin by ELISA. The level of FPA in the concentrated supernatants were determined by EIA technique.

E. Western blot analysis after treatment of FIBs with human thrombin

Samples were taken before the addition of thrombin (Time=0 mins) and the clot after polymerization were dissolved in reducing buffer. Samples were analyzed on a 8-25% phast gel in the presence of SDS. The results are shown in Figure 11.

Lanes 1 and 2 of Figure 11 show human fibrinogen reference protein in buffer at concentrations of 50 and 10 ngs before the addition of thrombin. Lane 3 shows the dissolved clot generated from hFIB at an initial concentration of 10 ng. Lane 4 shows transgenic whey pellet-2 FIB (30 ng applied to gel) before addition of thrombin. Lane 5 shows dissolved clot generated from transgenic whey pellet-2 FIB. Lane 6 shows mouse plasma derivative (30 ng total FIB cross reactive signal by ELISA, 1000 ng mouse FIB by silver-stain analysis using SDS-PAGE). Lane 7 shows samples from the incubation of mouse plasma derivative FIB with thrombin; this precipitate is not the cloudy gel-like precipitate seen

with TG whey (pellet-2) and hFIB spiked control whey (pellet-2).

5 The Western analysis under reducing conditions of the hFIB reference protein shows three distinct chains, and the dissolved clot resulting from thrombin digestion shows three chains with an apparent lower molecular mobility under reduced conditions. Release of  
10 fibrinopeptide A from the  $\alpha$  chain by incubation with thrombin results in a lower apparent molecular of the  $\alpha$  chain. Dissolved clots from transgenic whey (pellet-2) under reducing conditions gave two distinct bands with lower apparent molecular weights when compared to rhFIB before addition of thrombin. A mouse plasma sample gave bands which were reactive to FIB antisera and had lower  
15 apparent molecular weights than reduced hFIB.

F. Assay of fibrinopeptide A after thrombin digestion of rhFIB

The levels of FPA in the concentrated samples were determined by an EIA technique. Immobilon AV membranes  
20 were wetted in imidazole buffer for 10 minutes and dried for 60-90 minutes at room temperature. FPA standards ranging from 0.2-0.125 mg/ml and assay supernatant fluids were applied as 1  $\mu$ l spots onto a pre-wetted Immobilon AV membrane for the purpose of covalent immobilization.  
25 Smaller peptides such as FPA (MW 1,800) are kinetically favored for immobilization over larger peptides or milk proteins with molecular weights in excess of 10,000. The spots were allowed to dry at room temperature and the membrane was blocked with 0.5 % casein for 2 hours at the  
30 same temperature. Covalently bound FPA was detected with a sandwich of rabbit anti-FPA and anti-rabbit/HRP conjugate antibodies. Bound chromophore was detected with metal enhanced DAB substrate. The spots were scanned on a Shimadzu Densitometer at 270 nm. The results are shown  
35 in Figure 12.

Lanes 1-6 of Figure 12 show applications of FPA standards on membranes ranging from 200-6.25 ngs. Lane 7 shows 1  $\mu$ l application of transgenic whey (pellet-2)

before the addition of thrombin, and lane 8 shows the supernatant fluid from transgenic whey (pellet-2). Lane 9 shows 1  $\mu$ l application of nontransgenic (NTG) whey (pellet-2) before the addition of thrombin, and lane 10 shows the supernatant fluid from NTG (pellet-2). Lane 11 shows 1  $\mu$ l application of the hFIB reference protein before the addition of thrombin, and lane 12 shows the supernatant fluid from the hFIB reference protein. Lane 13 shows a 1  $\mu$ l application of mouse plasma (putative fraction from pellet-2) before the addition of thrombin, and lane 14 shows supernatant from mouse plasma.

hFIB in assay buffer when treated with thrombin released FPA as detected by a signal at 270 nm. The yield of FPA from hFIB in assay buffer treated with thrombin was  $100 \pm 10$  % based on three independent trials (Table 1). No detectable amount of FPA was released from control whey (pellet-2) treated with thrombin. 1,000 ng of mouse FIB from mouse plasma (equivalent to 25 ngs of FIB cross reactive signal) gave a FPA yield of 5%. Transgenic whey (pellet-2) and hFIB spiked control whey (pellet-2) when treated with thrombin released FPA in the supernatant fluid, and no residual rhFIB or hFIB was detected in the supernatant fluid by ELISA. The yield based on the amount of FPA released from TG whey (pellet-2) and hFIB spiked control whey (pellet-2) is estimated to be  $60 \pm 8$ % and  $75 \pm 9$ % respectively based on three independent trials. A 1/1 molar ratio of FPA to fibrinogen is assumed in the calculation.

TABLE 8  
Thrombin Treatment of Fibrinogen Samples

Sample (supernatant)	Fibrinogen $\mu\text{g/ml}$ (ELISA)		% FPA yield
	T = 0 min	T = 24 hours	T = 24 hours
hFIB (buffer)	100	0.1	100 $\pm$ 5
hFIB (buffer)	10	n.d.	100 $\pm$ 10
Spiked NTG whey (pellet-2)	50	0.1	75 $\pm$ 9
TG whey (pellet-2)	30	n.d.	60 $\pm$ 8
NTG whey (pellet- 2)	30	n.d.	n.d.
Mouse plasma	30	n.d.	5

#### G. Summary

Mouse FIB was found to have a higher apparent molecular weight under nonreducing conditions both by silver-stained SDS-PAGE gels and corresponding Western blot analyses using polyclonal anti-human fibrinogen antisera, than human FIB. In contrast, the separate  $\alpha$ ,  $\beta$  and  $\gamma$  chains of the mouse plasma FIB, as detected by gel analyses under reducing conditions, had slightly lower apparent molecular weights (about 1-5 kDa per chain) than human FIB. These data from reduced and nonreduced analyses indicate that the disulfide bridging and resultant SDS-sensitive conformation of the assembled mouse FIB is different than for the human counterpart. Furthermore, the cross-reactivity to anti-human fibrinogen signal was about 40-fold less than human or rhFIB in both ELISA and Western analysis assuming about 2g/l mouse FIB in mouse plasma reference as detected by silver stain gel. Mouse plasma FIB was also found in transgenic and control mouse milks at about 200 to 300  $\mu\text{g/ml}$ . After purification processing, all Zn-derived pellet-2 from nondoped control whey or mouse plasmas exhibited no detectable clot formation nor FPA formation after treatment with human thrombin. But lower apparent

molecular weight FIB chain reaction products may have been produced by treatment with thrombin under conditions of about 20-fold higher initial concentration of FIB substrate than used for human FIB-doped whey derivative or rhFIB samples. Both the absence of FPA and an obvious clot after treatment with human thrombin was expected. While mouse FIB undoubtedly has regions of well-conserved sequence homology, both the mouse FPA sequence and the nature of the digestion by human thrombin (slower reaction kinetics due to site specificity of human thrombin for human fibrinogen) is likely to be different. The 40-fold lower cross-reactivity of mouse FIB with anti-hFIB antisera is additional evidence of the differences in mouse and human fibrinogens.

The FIB doped into whey and recovered by the above purification process was readily converted to fibrin by the addition of human thrombin. A normal fibrin clot and stoichiometric release of FPA into the supernatant solution was found after treatment with thrombin. A characteristically lower apparent molecular weight of the fibrin chains from the clot was found relative to the starting FIB precursor chains.

The assembled rhFIB possessed an apparent molecular weight similar to that of human FIB reference standard. The physicochemical properties of the rhFIB were similar to those of hFIB as reflected by the similar partitioning during the  $Zn^{2+}$  selective purification scheme.  $Zn^{2+}$  is a coordinate covalent bond of proteins primarily through access to proximally portioned, surface positioned histidine residues, and thus the tertiary conformation of the proteins is changed by the formation of  $Zn^{2+}$  bonds Lonnerdal (1982), *supra*. Changes in tertiary confirmation by Zn-bonding are reflected in a Zn-concentration dependent manner by the precipitation (loss of solubility). Similar amounts of recombinant and human FIB precipitated out at 24 mM  $Zn^{2+}$  and as found in pellet-2. The sensitivity of this technique is seen with the structurally related mouse FIB which did not precipitate to any significant degree at this same level



of Zn. Thus, the presentation of surface histidine residues is similar between human and recombinant FIB, but much different for mouse FIB. The separate chains of rhFIB will slightly lower apparent molecular weights than non-recombinant human FIB due possibly to glycosylation of  $\beta$  and  $\gamma$  chains. The reactivity to anti-human fibrinogen antisera of purified recombinant fibrinogen was generally equivalent to human fibrinogen.

The rhFIB recovered from the transgenic was readily converted to fibrin by the addition of human thrombin and in a manner similar to that of human FIB reference protein. A normal human fibrin clot and stoichiometric release of FPA into the supernatant solution was found after treatment with human thrombin. The yield of FPA detected by EIA after treatment with human thrombin for rhFIB fibrinogen was equivalent to that obtained with human FIB. As the yields of fibrin from human or recombinant fibrinogens were high, the residual milk proteins present after purification did not interfere significantly with the thrombin-fibrinogen reaction. A characteristically lower apparent molecular weight of the recombinant human fibrin chains from the clot was found relative to the starting FIB precursor chains.

In summary, these analyses have shown that the rhFIB made has a genuine and native structure with respect to biological activity. This was evidenced by its simultaneous consumption and conversion into a normal fibrin clot while acting as a substrate during enzymatic reaction with human thrombin, stoichiometric release of native fibrinopeptide A upon formation of fibrin, lower relative molecular weights of resultant fibrin polypeptide chains relative to FIB precursors, its coordinate chemical bonding properties with Zn, and its equivalent reactivity with human antisera relative to a human FIB reference protein.



SEQUENCE LISTING

GENERAL INFORMATION:

- (i) APPLICANT: Velandar, William H.  
Lord, Susan T.  
Drohan, William N.  
Lubon, Henryk  
Johnson, John L.  
Russell, Christopher G.
- (ii) TITLE OF INVENTION: TRANSGENIC FIBRINOGEN
- (iii) NUMBER OF SEQUENCES: 12
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Foley & Lardner  
(B) STREET: 3000 K Street, N.W., Suite 500  
(C) CITY: Washington, D.C.  
(E) COUNTRY: USA  
(F) ZIP: 20007-5109
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
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- (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: BENT, Stephen A.  
(B) REGISTRATION NUMBER: 29,768  
(C) REFERENCE/DOCKET NUMBER: 30523/115/ANRC
- (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: (202)672-5300  
(B) TELEFAX: (202)672-5399  
(C) TELEX: 904136

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCCAGCTGC AGCCATGAAG

20

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCCAGGGAT ATCCCTGCAG CCATGAAG

28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCTAGGTACC ATGTTTCCA TGAGGATCGT

30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATTAGAGC GGGACAGATC CCATGGTGAC

30

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTAGGTACC ATGAAAAGAA TGGTTTCGTG

30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAAGAAGGG TGTCGTTATC CCATGGTGAC

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTAGGTACC ATGAGTTGGT CCTTGCACCC

30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTGTCCGACC TCTGCAAATT CCATGGTGAC

30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGTGTGGCC AAGAAGGAAG TGTTG

25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GATGTCTTTC CACAACCCTT GGGC

24

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCGATAGCC ACCTCCTCTG ATG

23

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCTGGACTTC AAAGTAGCAG CGTC

24